Development of small molecule antagonists of androgen receptor for prostate cancer treatment

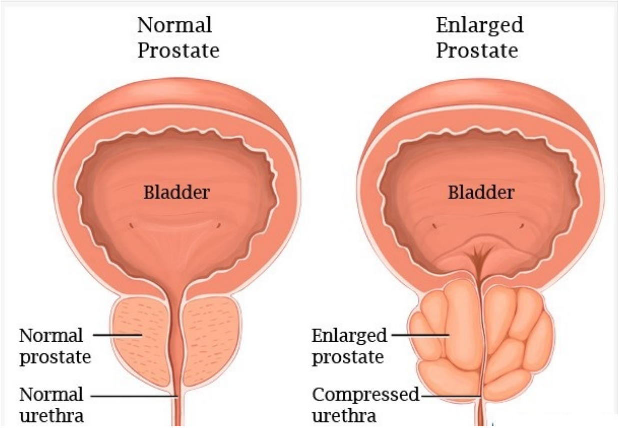
Wipf Group I University of Pittsburgh

Serene Tai

Research Topic Seminar

1st April 2017

Prostate Cancer – Facts and Statistics



❖ **Prostate**

- compound tubuloalveolar exocrine gland of the male reproductive system
- about the size of a walnut

❖ **Prostate cancer**

- uncontrolled growth of cells in the prostate gland
- prostate adenocarcinoma is the major cancer type
- 1 in 7 men will be diagnosed with prostate cancer in their lifetime
- average age 66; rare before 40

❖ **Symptoms**

- no symptoms in the early stage
- pain and blood in urination, frequent urination, pain in bones and other areas occur in advanced stages

American Cancer Society, Cancer Facts & Figures 2017

Prostate Cancer – Facts and Statistics

Figure 3. Leading Sites of New Cancer Cases and Deaths – 2017 Estimates

Estimated New Cases

Male			Female		
Prostate	161,360	19%	Breast	252,710	30%
Lung & bronchus	116,990	14%	Lung & bronchus	105,510	12%
Colon & rectum	71,420	9%	Colon & rectum	64,010	8%
Urinary bladder	60,490	7%	Uterine corpus	61,380	7%
Melanoma of the skin	52,170	6%	Thyroid	42,470	5%
Kidney & renal pelvis	40,610	5%	Melanoma of the skin	34,940	4%
Non-Hodgkin lymphoma	40,080	5%	Non-Hodgkin lymphoma	32,160	4%
Leukemia	36,290	4%	Leukemia	25,840	3%
Oral cavity & pharynx	35,720	4%	Pancreas	25,700	3%
Liver & intrahepatic bile duct	29,200	3%	Kidney & renal pelvis	23,380	3%
All sites	836,150	100%	All sites	852,630	100%

Estimated Deaths

Male			Female		
Lung & bronchus	84,590	27%	Lung & bronchus	71,280	25%
Colon & rectum	27,150	9%	Breast	40,610	14%
Prostate	26,730	8%	Colon & rectum	23,110	8%
Pancreas	22,300	7%	Pancreas	20,790	7%
Liver & intrahepatic bile duct	19,610	6%	Ovary	14,080	5%
Leukemia	14,300	4%	Uterine corpus	10,920	4%
Esophagus	12,720	4%	Leukemia	10,200	4%
Urinary bladder	12,240	4%	Liver & intrahepatic bile duct	9,310	3%
Non-Hodgkin lymphoma	11,450	4%	Non-Hodgkin lymphoma	8,690	3%
Brain & other nervous system	9,620	3%	Brain & other nervous system	7,080	3%
All sites	318,420	100%	All sites	282,500	100%

Estimates are rounded to the nearest 10, and cases exclude basal cell and squamous cell skin cancers and in situ carcinoma except urinary bladder.

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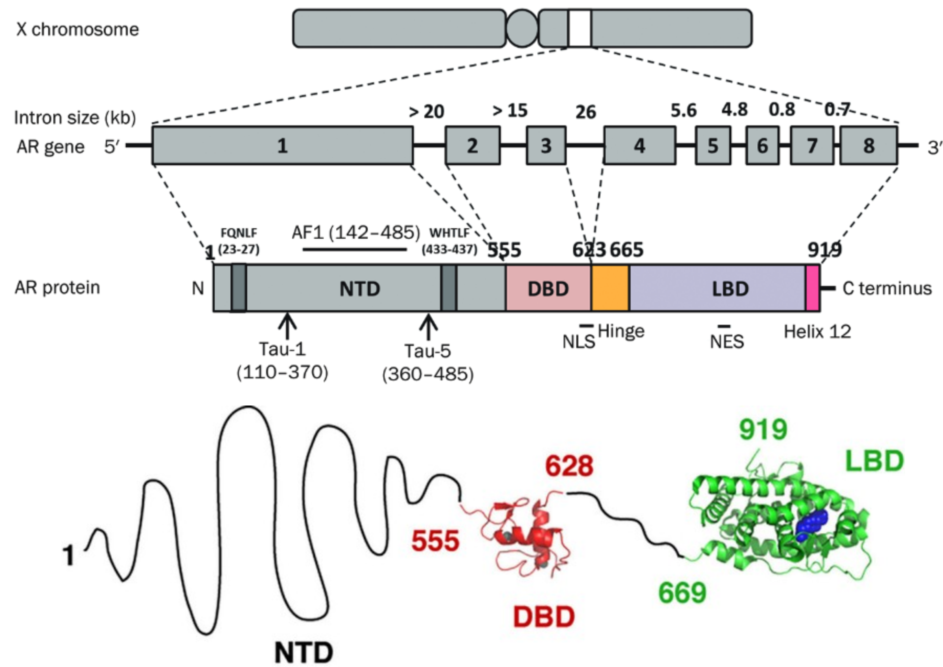
Androgen Receptor (AR)

- AR is a ligand-dependent nuclear transcription factor that belongs to the steroid hormone receptor superfamily
- AR gene encodes a protein of 919 amino acids (110 kDa)
- Consists of 4 major functional domains:
  - N-terminal domain (NTD)
  - DNA binding domain (DBD)
  - Hinge region
  - Ligand binding domain (LBD)
- Structure of full length AR has not been solved



Acta Pharmacologica Sinica. **2015**, *36*, 3-23
J. Carcinog. **2011**, *10*, 20
ACS Chem. Biol. **2016**, *11*, 2499–2505

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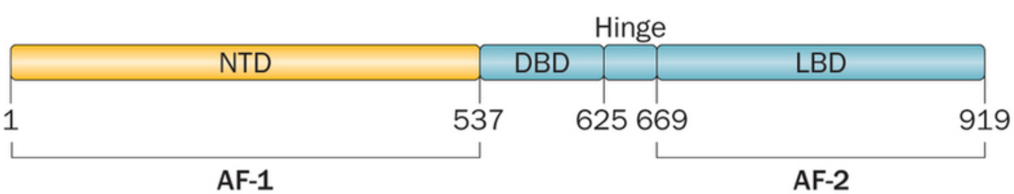
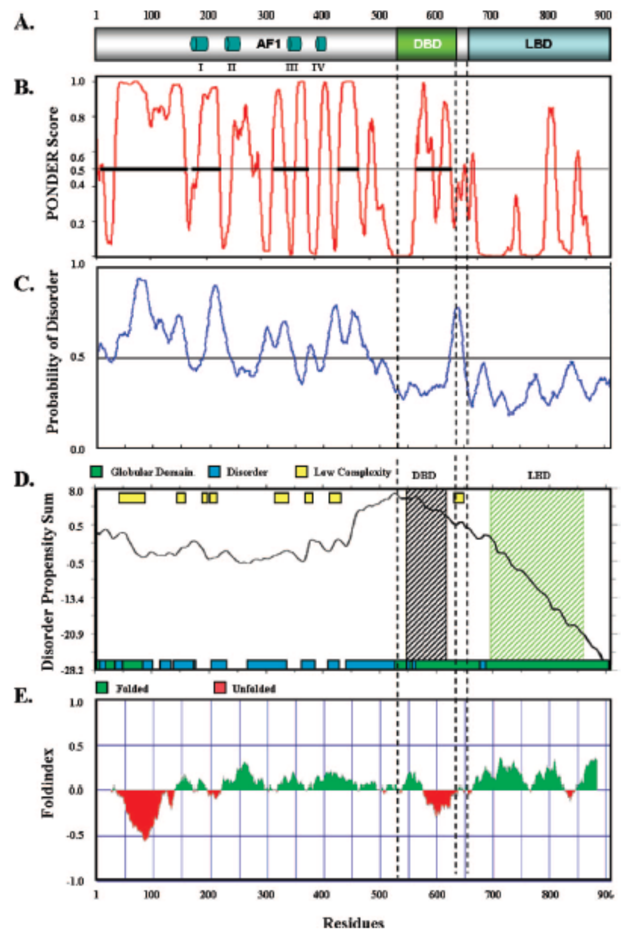
N-terminal Domain (NTD)

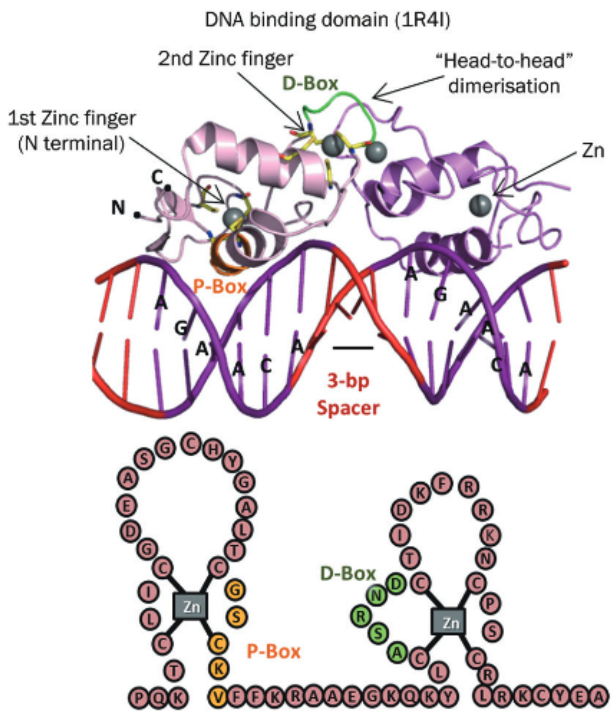
- Accounts for more than half the size of AR
- Contains transactivation function (AF1)
- The polyglutamine (polyQ - CAG) and polyglycine (polyG - GGC) tracts affect AR transactivation activity
- A combination of experimental and computational analysis suggest that the NTD exists as partially folded protein intermediate (neither full random coil nor stable globular comformation)
- Flexible for binding with multiple structurally diverse protein partners

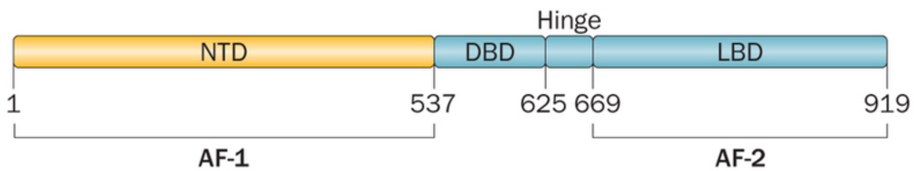




Biochem. **2008**, *47*, 3360-3369

DNA Binding Domain (DBD) and Hinge





- ❖ Highly conserved cysteine rich region
- ❖ Monomer consists of two zinc finger domains
  - P-Box: coordinates contacts with DNA groove at the promoter region
  - D-Box: functions as DBD/DBD binding site in AR dimer
- ❖ **Hinge region**
  - contains part of the nuclear localization signal (NLS) for AR nuclear transport
  - nuclear import is mediated by importin-α

Acta Pharmacologica Sinica. **2015**, *36*, 3-23
Nat. Rev. Urology. **2015**, *12*, 37-47

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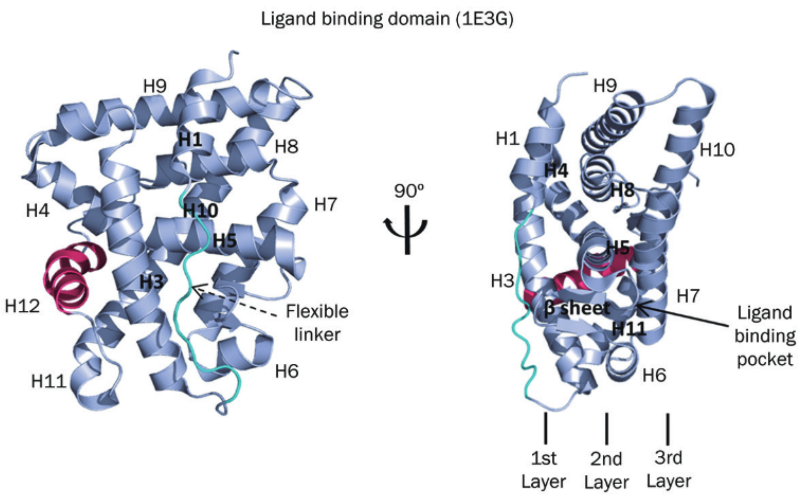
Ligand Binding Domain (LBD)



Structure

- ❖ Arranged in a 3-layer, antiparallel α-helical sandwich fold
- ❖ Consists of 11 α-helixes and 4 short β-strands
- ❖ The ligand binding pocket (LBP) is surrounded by H3, H5, and H11
- ❖ H12 forms the core of the activation function 2 (AF2) domain

Functions

- ❖ Binding site of androgens for AR signaling
- ❖ Ligand-dependent AF-2 interacts with NTD (helps stabilize AR dimer complex) and binds with co-activators during AR action
- ❖ Regulation of AR nuclear export
- ❖ Most popular target for AR antagonists

J. Biol. Chem. **2000**, *275*, 26164-26171
Acta Pharmacologica Sinica. **2015**, *36*, 3-23

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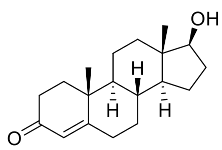
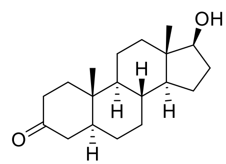
Androgens & AR Agonists

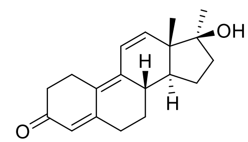
- The two most important endogenous androgens are testosterones and dihydrotestosterones (DHT)
- Synthesized in the testes (<95 %) and adrenal gland



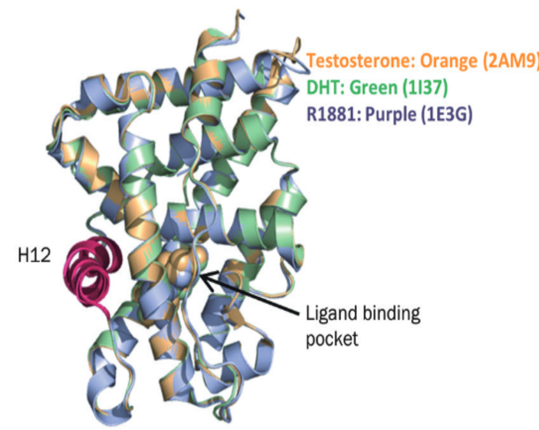
Testosterones

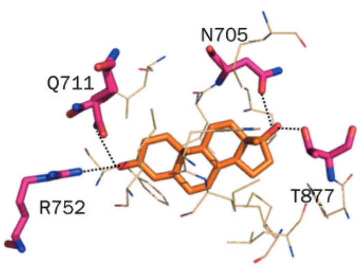


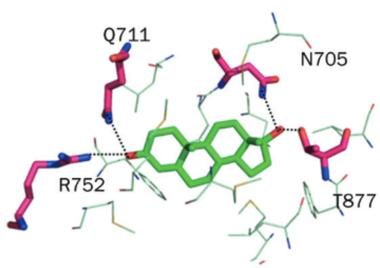
DHT

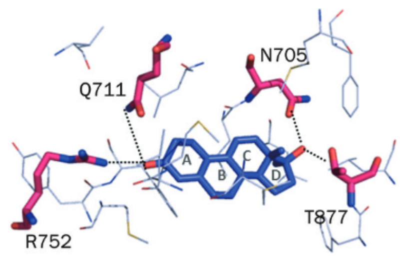


R1881



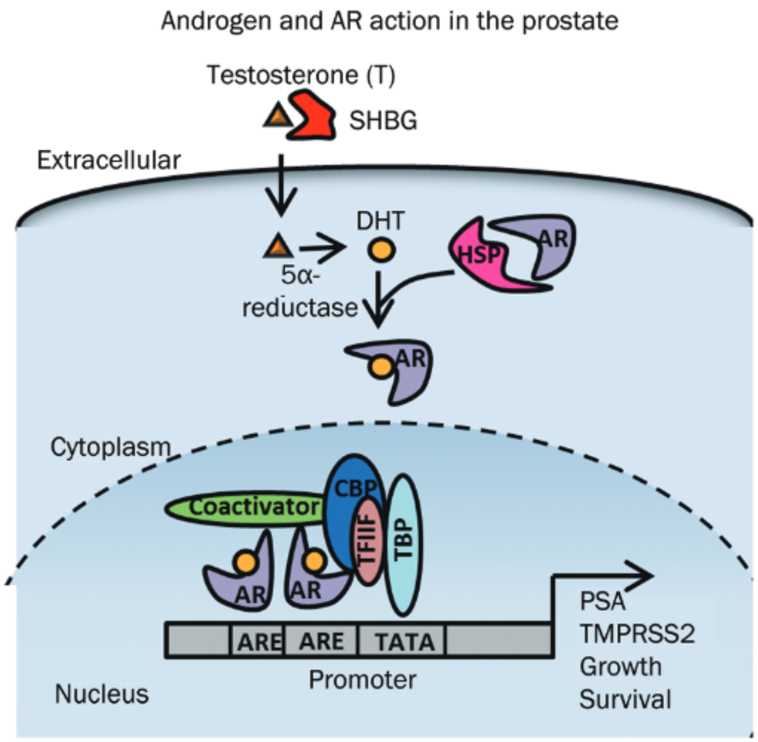


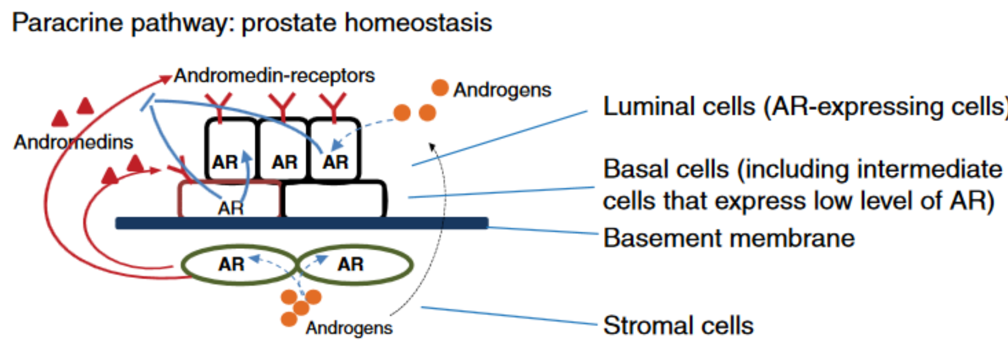




J. Biol. Chem. **2000**, *275*, 26164-26171
Proc. Natl. Acad. Sci. **2001**, *98*, 4904-4909
Protein Sci. **2006**, *15*, 987-999

Androgens & AR in Prostate Development

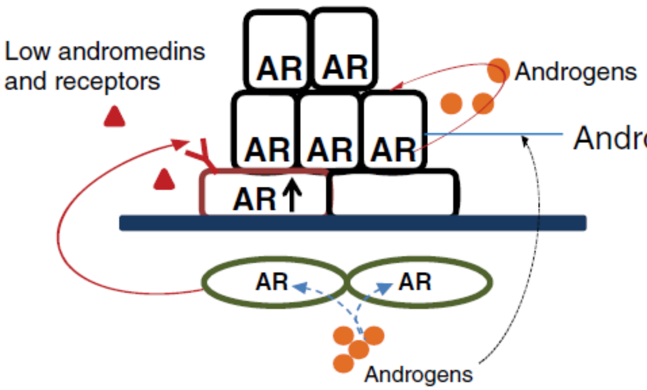




J. Mol. Endocrinol. **2015**, *54*, 15-29
Acta Pharmacologica Sinica. **2015**, *36*, 3-23

Androgens & AR in Prostate Cancer

- Autocrine pathway: AR malignancy switch



- 159 out of 1029 mutations found in gene that encodes AR predispose males to prostate cancer
- A shorter polyQ length (CAG) in the NTD of the AR is associated with higher risk in prostate cancer
- Low serum testosterone level is correlated to increased cancer risk
- A prostate specific antigen (PSA) level above 4 ng/mL is considered abnormal

Hum. Mutat. **2012**, *33*, 887-894
J. Mol. Endocrinol. **2015**, *54*, 15-29
Acta Pharmacologica Sinica. **2015**, *36*, 3-23

Initiation of Prostate Cancer

❖ **Alterations in the AR-driven transcriptional program**

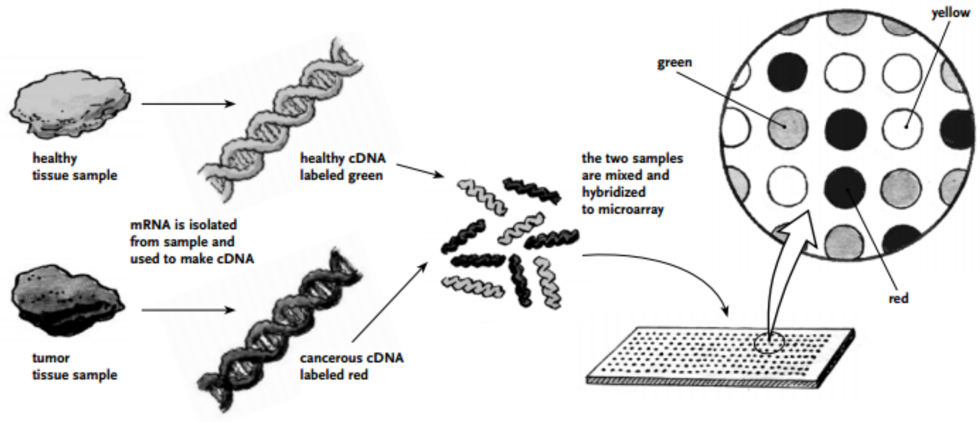
- **DNA microarray** analysis and **high-throughput profiling**

    (i) identification of androgen-regulated gene

    (ii) analysis of androgen-regulated gene expression levels in normal vs cancer cell lines

-Eg: characterization of the temporal program of transcription identified 146 androgen-responsive genes from LNCaP cancer cell lines with transcript alterations

-Eg: FKBP51 androgen-regulated gene was expressed significantly higher in tumor samples relative to benign samples



PNAS. **2002**, *99*, 11890-11895
Endocrinology. **2004**, *145*, 3913-3924

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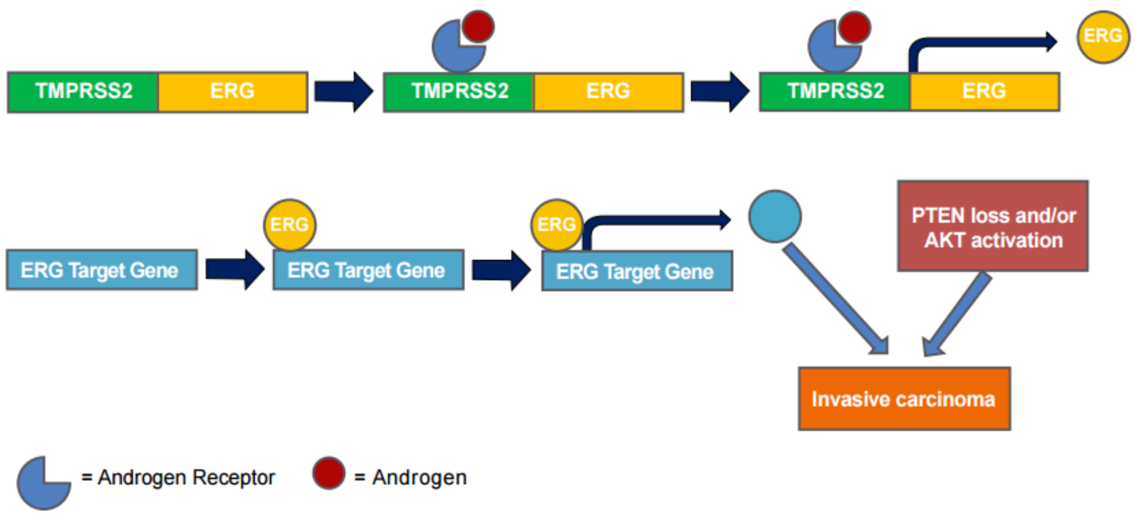
Initiation of Prostate Cancer

❖ **Changes in the interaction of AR with AR cofactors**

- **Chromosomal fusions** of androgen-regulated promoter, transmembrane protease, serine 2 (TMPRSS2), with AR coregulators (ERG and ETV1) from the E-twnty-six (ETS) family have been found in >50% of the patients in early stage of prostate cancer



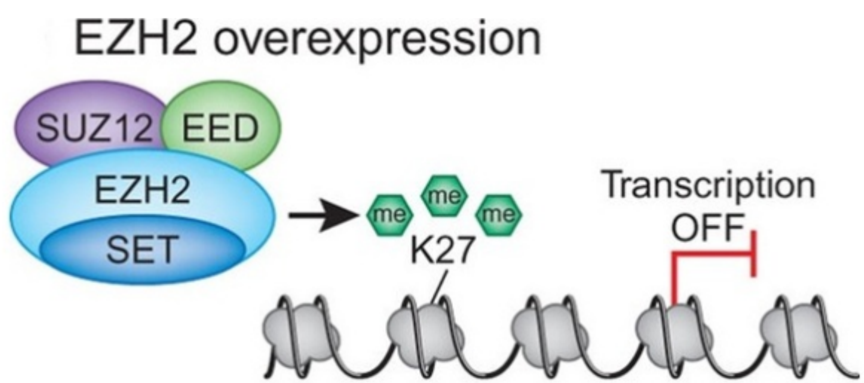
J. Cancer. Sci. Ther. **2012**, *4*, 94-101
Nat. Rev. Cancer. **2014**, *145*, 187-198

Initiation of Prostate Cancer

- **Post-translational modifications contributes to cell proliferation**
  - An extensive list of genes that exhibits hypermethylation has been reported to occur at the earlier stage of prostate cancer
  - Overexpression of enhancer of zeste homolog 2 (EZH2), an epigenetic modifier, is linked to the trimethylation of H3K27

- EZH2 was recently found to be an coactivator of AR when it is phosphorylated
  - Phosphorylation of EZH2 suppresses the methylation of H3K27

- The coordinate regulation of AR and EZH2 activity still needs to be explored



Nat. Rev. Cancer. **2014**, *145*, 187-198

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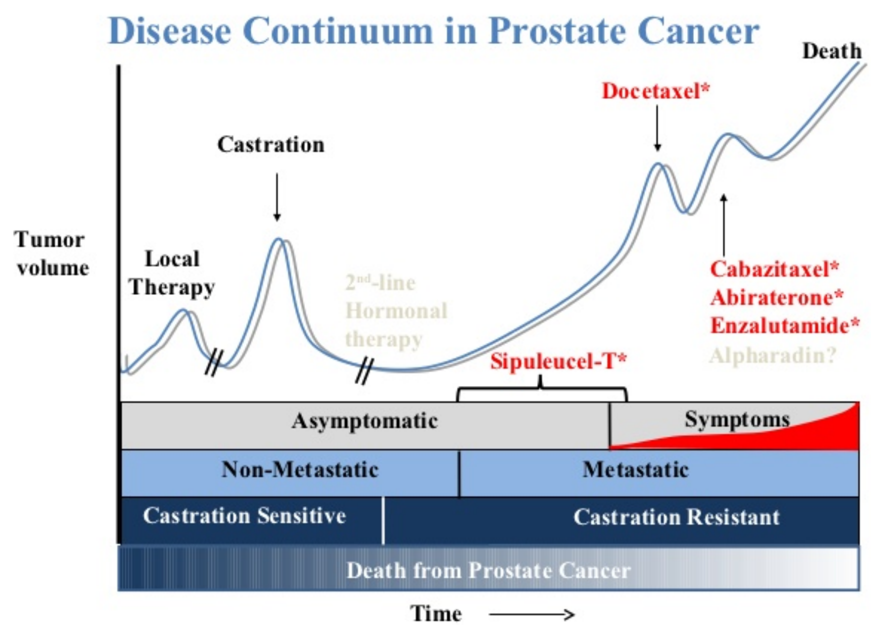
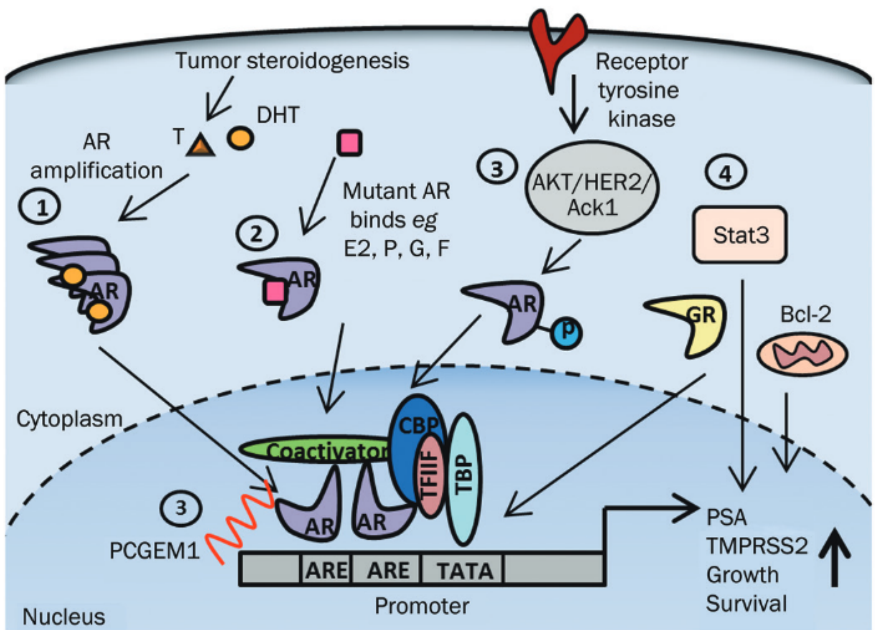
Castration-Resistant Prostate Cancer (CRPC)

- CRPC is an incurable stage of cancer, where the cancers became resistant to hormone therapy and resumed growth
- Previously known as hormone-refractory prostate cancer or androgen-independent prostate cancer
- >90 % of patients with CRPC developed metatheses
- Mean survival time 1-2 years



Cur. Oncor. **2010**, *17*, 72-79

Proposed Mechanisms of CRPC



- ❖ AR overexpression & continued tumor steroidogenesis
- ❖ Promiscuous binding and activation of mutant AR by alternative ligands
- ❖ Ligand-independent AR activation via crosstalk with other signaling pathways
- ❖ Complete AR-independent mechanisms

Acta Pharmacologica Sinica. **2015**, *36*, 3-23

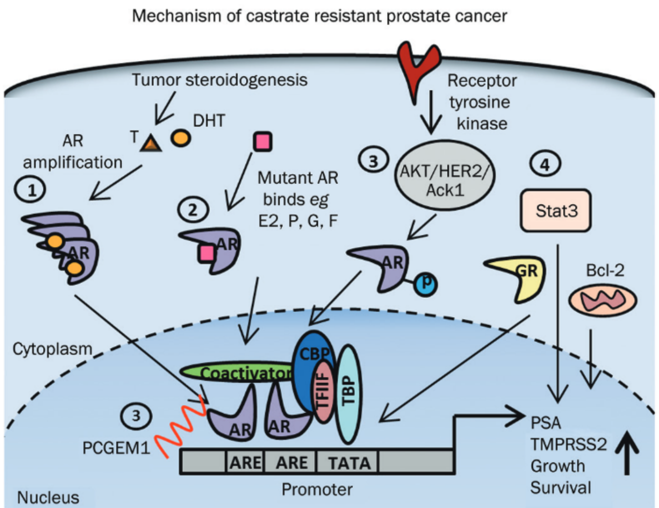
Proposed Mechanisms of CRPC

❖ **AR overexpression & continued tumor steroidogenesis**

- Studies showed that 28% of androgen-independent tumors that developed after hormone therapy had increased AR expression due to AR gene amplification
- Androgens from *in situ* tumoral synthesis and residual adrenal synthesis
- Decreased level of androgen inactivating enzymes
- CRPC cells became more sensitive due to the lower threshold of androgens

❖ **Promiscuous binding and activation of mutant AR by alternative ligands**

- Amino acid substitution from AR mutations at the LBD decreased ligand selectivity and specificity
- AR mutations could also caused AR antagonists to induce an agonist role



Acta Pharmacologica Sinica. **2015**, *36*, 3-23

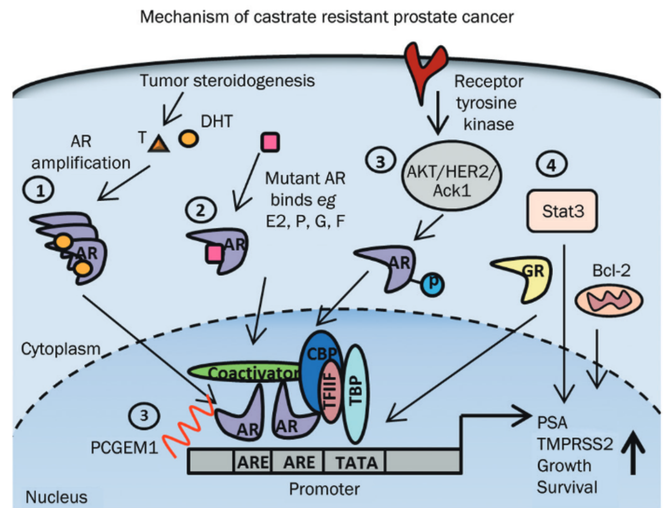
Proposed Mechanisms of CRPC

❖ **Ligand-independent AR activation via crosstalk with other signaling pathways**

- AR activation via phosphorylation by AKT, HER2, and Ack1 kinases
- AR activation by binding with long non-coding RNA

❖ **AR-independent mechanisms (bypass pathway)**

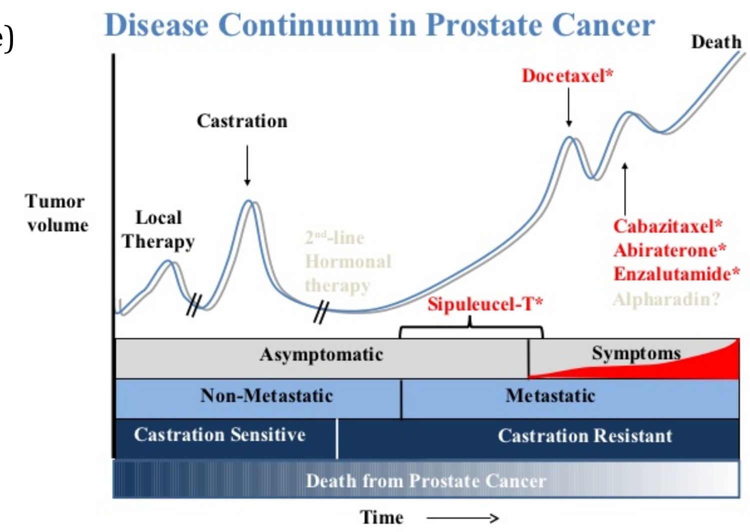
- Inflammatory response triggered by dying cells causes infiltration of B and T cells
- Upregulation of Stat3 signaling and anti-apoptotic protein Bcl-2
- Upregulation of glucocorticoid receptor (GR) drives the expression of a subset of AR target genes necessary for cell survival



Acta Pharmacologica Sinica. **2015**, *36*, 3-23

Treatments for Prostate Cancer & CRPC

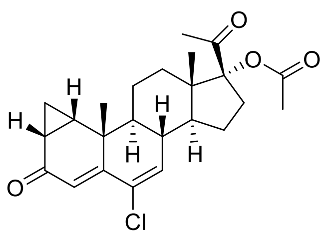
- Watchful waiting or active monitoring
- Radiation therapy
- Radical prostatectomy
- 1st line hormone therapy (orchiectomy, luteinizing hormone-releasing hormone (LHRH) agonists and antagonist)
- 2nd line hormone therapy (flutamide, bicalutamide, nilutamide)
- Abiraterone & enzalutamide – castrate-resistant treatment
- Chemotherapy (docetaxel, cabazitaxel, mitoxantrone, estramustine)



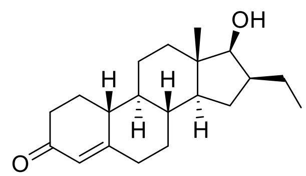
American Cancer Society

Androgen Deprivation Therapy (ADT)

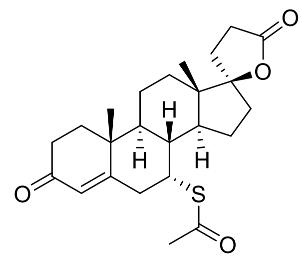
- Use of steroidal and/or non-steroidal hormones to reduce androgen level and inhibit AR function the prostate cancer treatment
- Discovery of chemical castration using estrogen by Charles Huggins published in 1941 won him the 1966 Nobel Prize in Physiology or Medicine
- Steroidal antiandrogens are rarely used due to off-target actions



Cyproterone acetate



Oxendolone



Spironolactone

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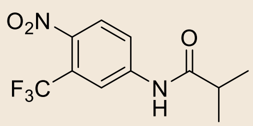
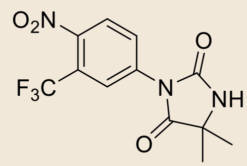
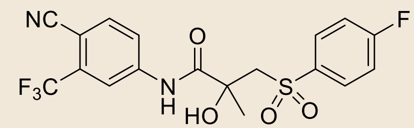
Non-steroidal Antiandrogens



Flutamide



Nilutamide



Bicalutamide

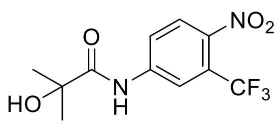
- The first non-steroidal antiandrogen (NSAA) marketed in 1983
- $T_{1/2}$: 5 – 6 hours
- IC_{50}: 1.3 μM
- Dosage: 750 – 1000 mg/day
- Side effects: diarrhea, hepatotoxicity

- Developed by Rousel and marketed in 1987 in Europe
- $T_{1/2}$: ~2 days
- IC_{50}: 0.41 μM
- Dosage: 300 mg/day
- Side effects: nausea, insterstitial pneumonitis, alcohol intolerance, visual disturbance

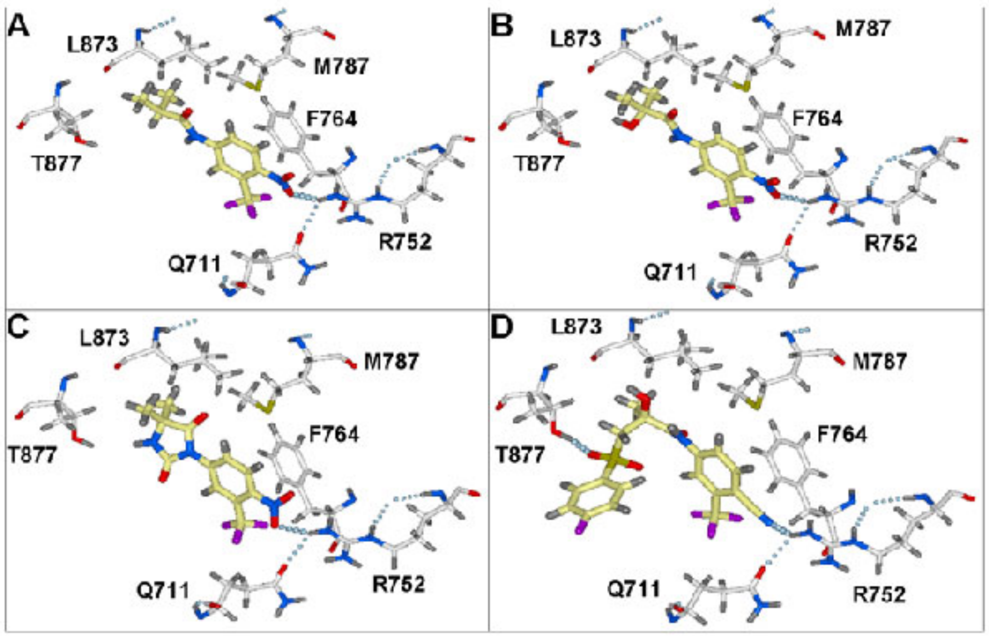
- Patented in 1982 and marketed in 1995 by AstraZaneca
- $T_{1/2}$: ~7 days
- IC_{50}: 0.16-0.23 μM
- Dosage: 50 - 150 mg/day
- Side effects: fatigue

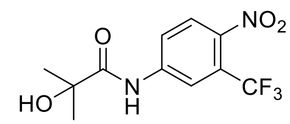
Cancer cells developed resistance toward these drugs and demonstrated agonist properties

Computational Modeling

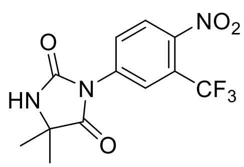


Flutamide

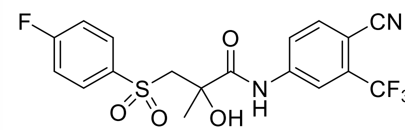




Hydroxyflutamide



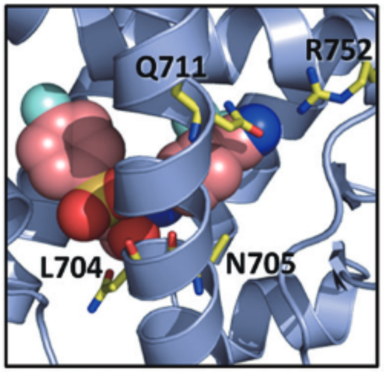
Nilutamide

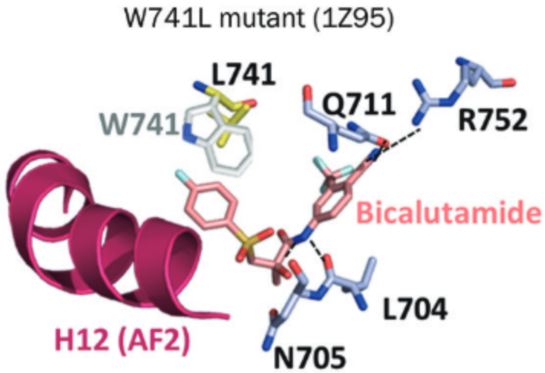


Bicalutamide

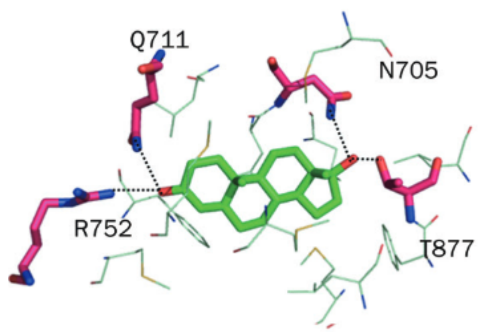
PNAS. **2007**, *104*, 11927-11932

Bicalutamid-bound AR LBD W741L





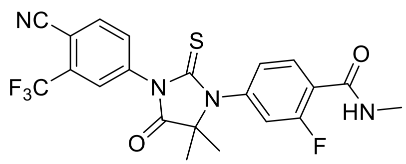
- Two LBD mutations, W741L and W741C, were discovered during bicalutamide treatment
- CN group forms H-bonding with Q711 and R752
- Amide nitrogen and chiral –OH form H-bonding with L704 and N705
- Mutation: bulky W741 is replaced by L741, more space to accommodate 4-fluorophenyl ring of bicalutamide



DHT-bound AR WT

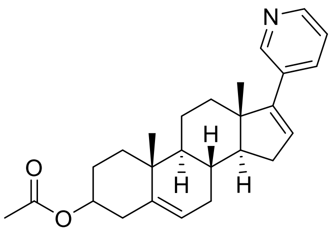
PNAS. **2005**, *102*, 6201-6206

Enzalutamide & Abiraterone



Enzalutamide

- Developed by Jung (UCLA) and Sawyer (MSK) and marketed by Medivation in 2012
- 2nd Gen CRPC drug: +5 months survival
- $T_{1/2}$: 8-9 days
- IC_{50}: 21-36 nM
- Dosage: 160 mg/day
- Side effects: seizures

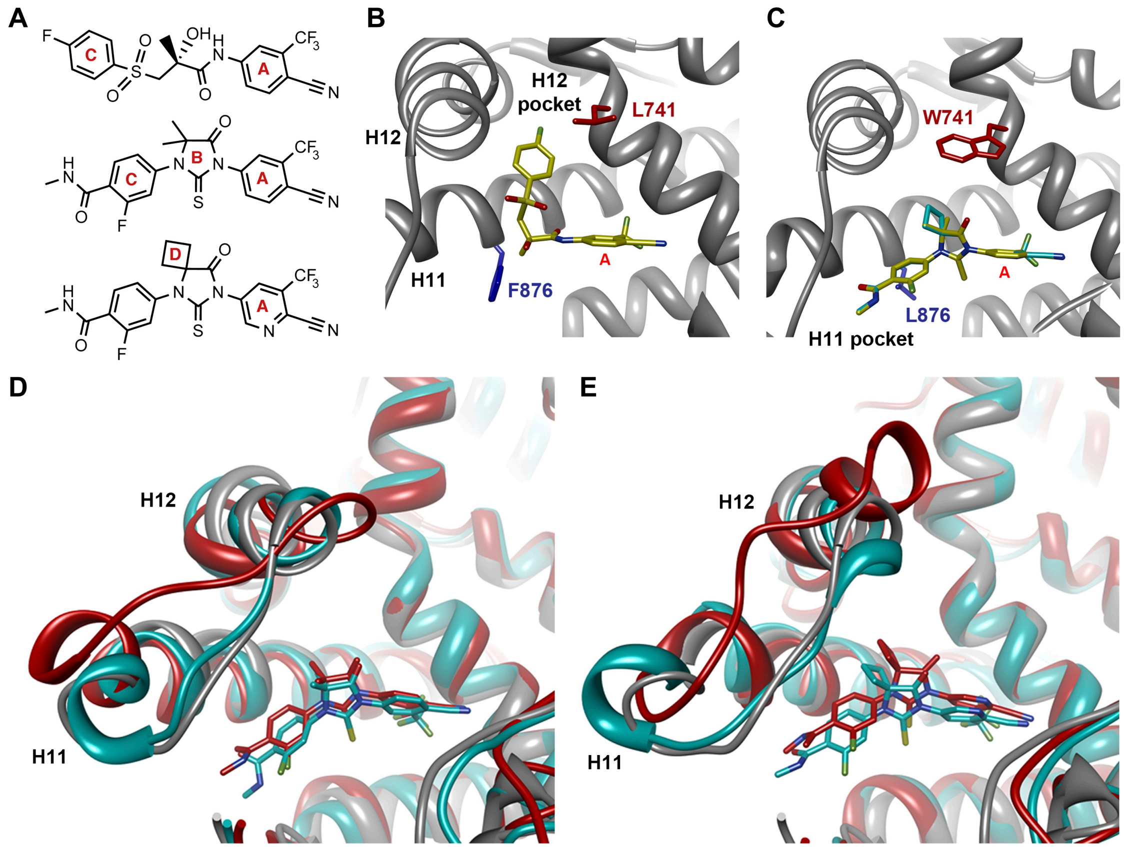


Abiraterone Acetate

- Developed in the 1990s and marketed by Johnson&Johnson in 2009
- Steroidal CYP17A1 inhibitor (inhibits androgen synthesis)
- $T_{1/2}$: 12-18 h
- IC_{50}: 2.5 - 15 nM
- Dosage: 1000 mg/day
- Side effects: urinary tract infection, diarrhea, hypertension

Enzalutamide Computational Modeling

A. Bicalutamide (top), enzalutamide (middle), ARN-509 (bottom)
B. Ligand docking of bicalutamide (gold) in AR W741L (gray)
C. Ligand docking of enzalutamide (gold) and ARN-509 (cyan) in AR F876L (gray)
D. MD simulation at 10ns for enzalutamide with AR WT (red) and ARF876L (cyan)
E. MD simulation at 10ns for ARN-509 with AR WT (red) and ARF876L (cyan)



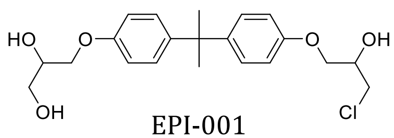
Elife. **2013**, 2, e00499

Targeting the NTD & DBD

❖ **Targeting the NTD**

- Unstructured NTD makes it a difficult target

- The constitutively active AR splice variants that lack the LBD are reported to be found in prostate cancer patients



EPI-001

- Targets the NTD AF-1 domain
- Blocks N/C interaction
- Blocks interaction of AF-1 with coactivator

❖ **Targeting the DBD**

- Limited examples due to specificity

Cancer Cell. **2010**, *17*, 535-546

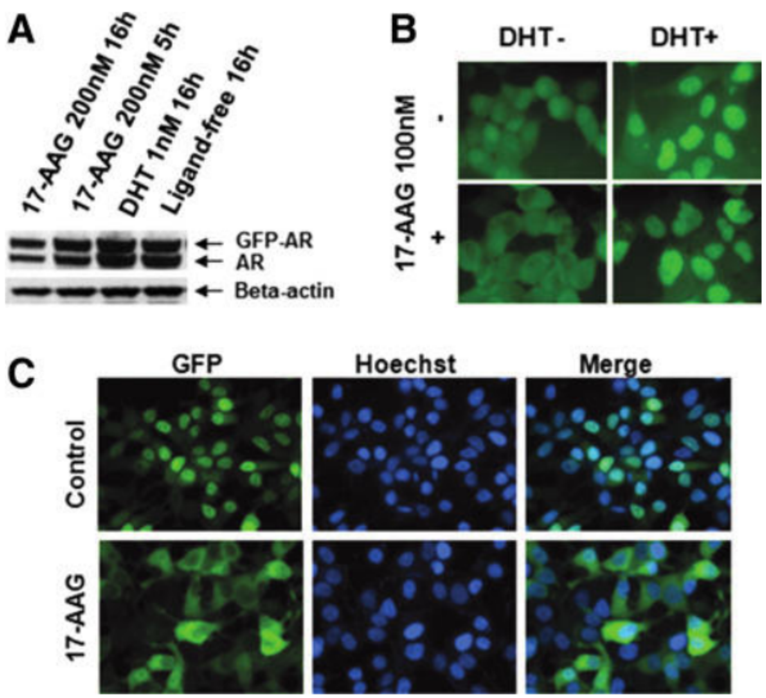
Wipf Group Strategy

High-throughput Screening Assay to Identify Inhibitors of AR Nuclear Localization in CRPC

26

High-throughput High-content Screening (HCS)

❖ Selection of 2GFP-AR expressing cell lines in the C4-2 CRPC cell background



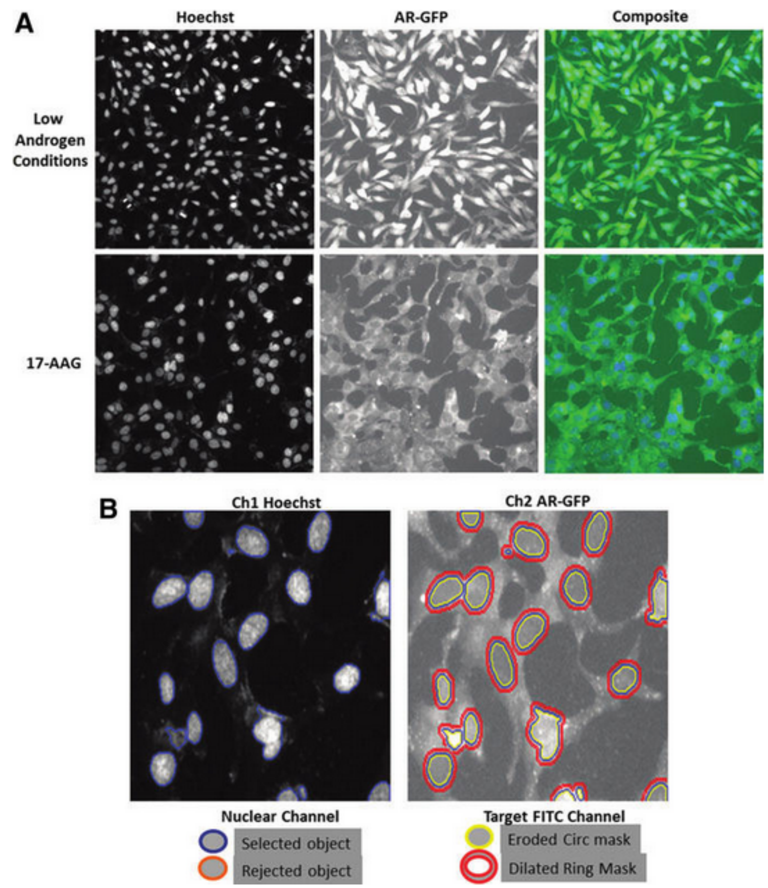
A. Effects of 17-AAG on 2GFP-AR and endogenous AR in the C4-2 cells
B. Effects of 17-AAG on the nuclear localization of 2GFP-AR in the C4-2 cells
C. Effects of 17-AAG on the nuclear localization of 2GFP-AR compared to endogenous AR in the C4-2 cells

ASSAY and Drug Development Technologies. **2016**, *14*, 226-239

High-throughput High-content Screening (HCS)

❖ 2GFP-AR nuclear localization HCS assay optimization

A. Grayscale images of Hoechst-stained nuclei and 2GFP-AR expression in N3 C4-2-2GFP-AR cells
B. Nucleus and cytoplasm masks generated by the molecular translocation (MT) image analysis segmentation

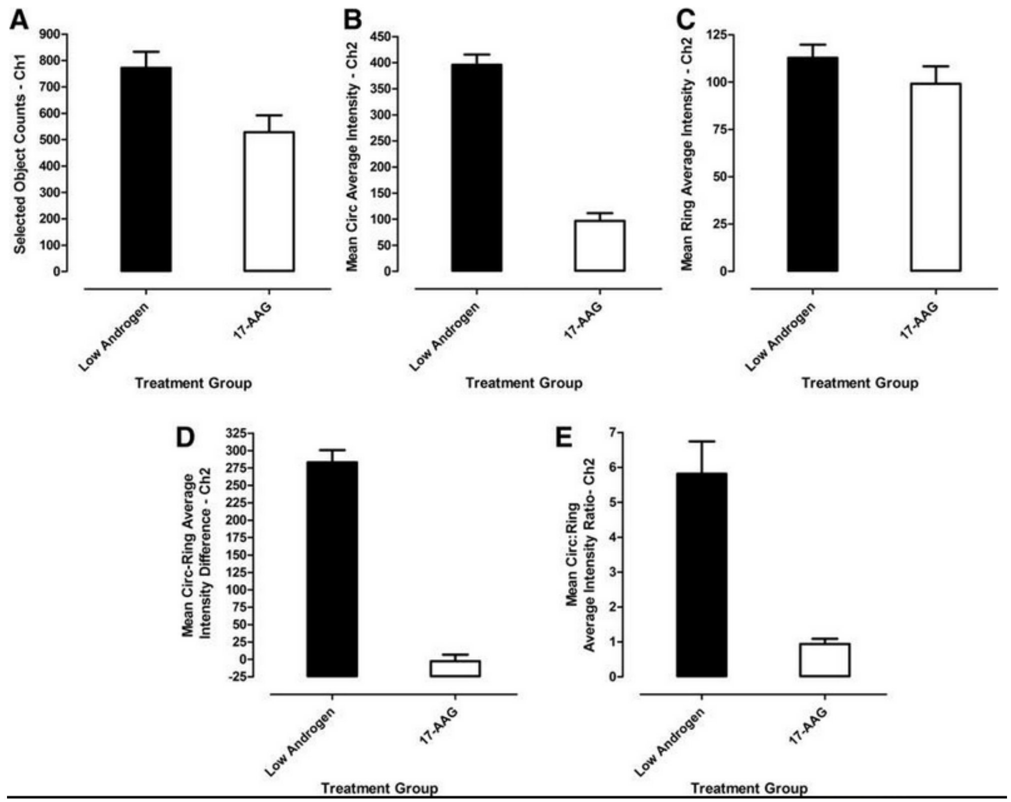


ASSAY and Drug Development Technologies. **2016**, *14*, 226-239

High-throughput High-content Screening (HCS)

❖ 2GFP-AR nuclear localization HCS assay optimization

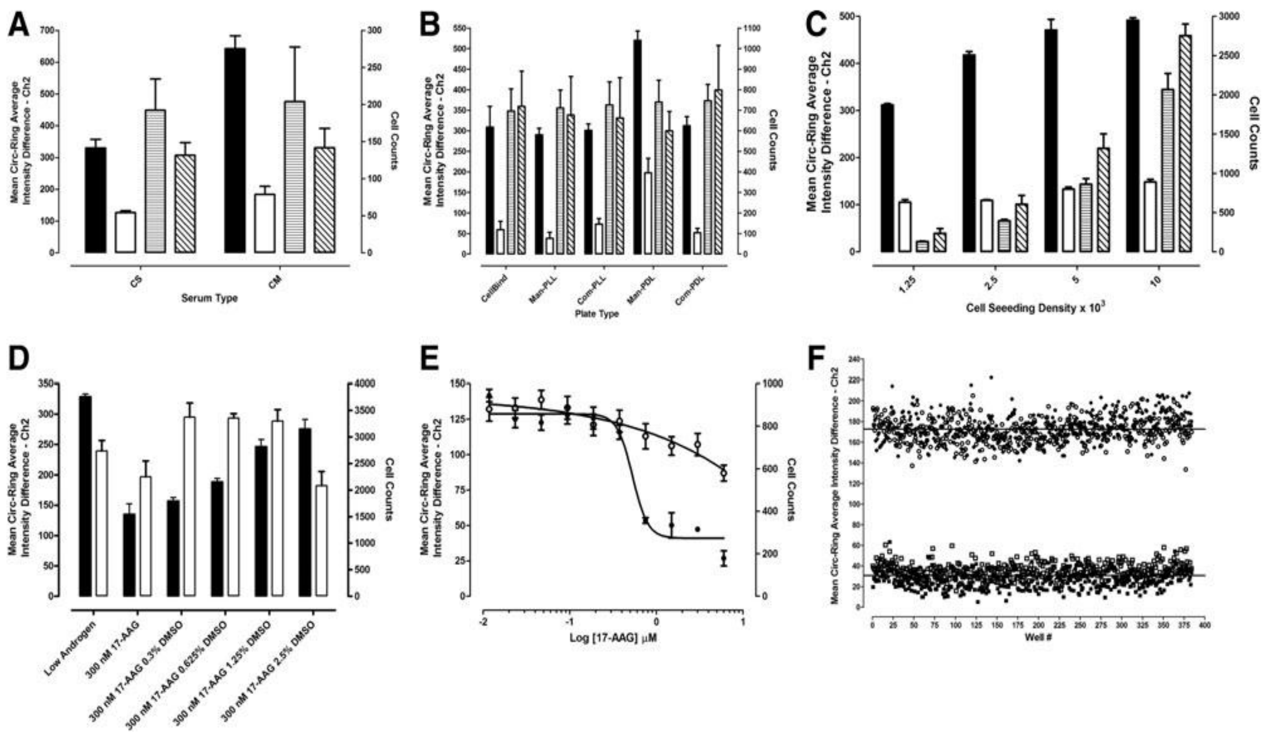
A. Selected object counts per image
B. Average 2-GFP-AR intensity in the nuclear 'circ' area
C. Average 2-GFP-AR intensity in the cytoplasmic 'ring' mask area
D. Mean (circ – ring) 2-GFP-AR average intensity difference
E. Mean circ:ring 2-GFP-AR average intensity ratio



ASSAY and Drug Development Technologies. **2016**, *14*, 226-239

High-throughput High-content Screening (HCS)

❖ 2GFP-AR nuclear localization HCS assay optimization



A. Charcoal stripped vs complete medium

B. Microtiter plate type

C. Cell-seeding density

D. DMSO tolerance

E. 17-AAG concentration response

F. 3-day variability and Z-factor coefficient

ASSAY and Drug Development Technologies. **2016**, *14*, 226-239

High-throughput High-content Screening (HCS)

- First high-throughput screening to identify small molecules capable of reducing AR nuclear localization in CRPC cells

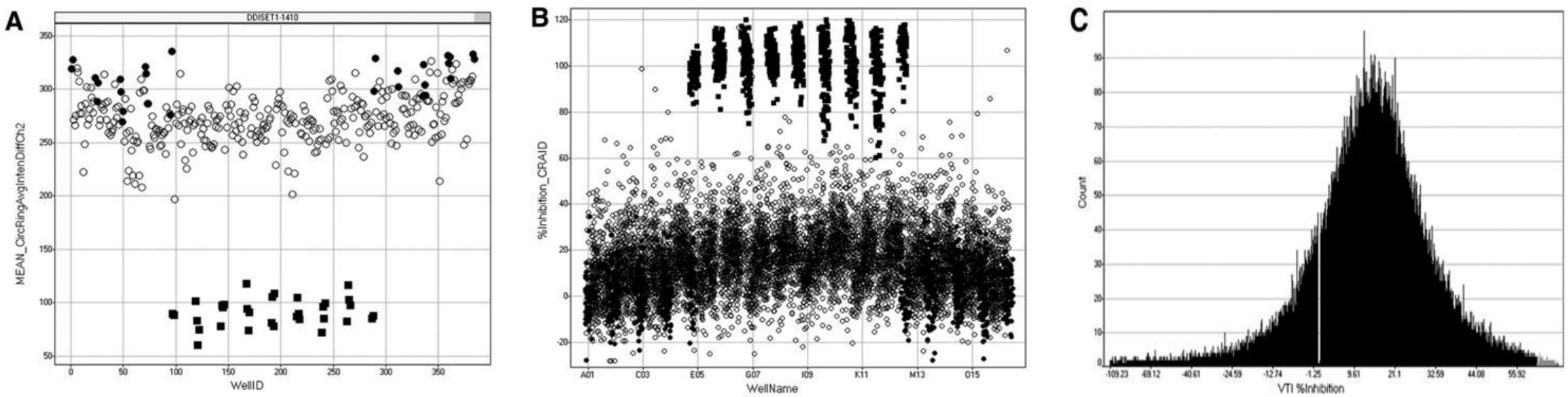
Table 1. Androgen Receptor Nuclear Localization HCS Assay Protocol

Step	Parameter	Value	Description
1	Plate cells	60 µL	3,000 N3 C4-2-2GFP-AR cells
2	Incubate cells overnight	16–24 h	Culture medium at 37°C, 5% CO_2, and 95% humidity
3	Library compounds/DMSO/DMSO +17-AAG to controls wells	20 µL	Compounds –20 µM final concentration in well +0.2% DMSO; controls –0.2% DMSO or 0.2% DMSO plus 3.0 µM 17-AAG
4	Incubate assay plates overnight	16–24 h	At 37°C, 5% CO_2, and 95% humidity
5	Aspirate media and fix cells	50 µL	3.7% formaldehyde containing 2 µg/mL Hoechst 33342 in PBS without Ca2+ and Mg2+, prewarmed to 37°C
6	Incubate plates	10–30 min	Ambient temperature
7	Aspirate fixative and wash 2× with PBS	50 µL	Fixative was aspirated and plates were then washed twice with 50 µL PBS without Ca2+ and Mg2+, 50 µL PBS in well
8	Seal plates	1×	Sealed with adhesive aluminum plate seals
9	Acquire images	10×, 0.3NA objective	Images of the Hoechst (Ch1) and AR-GFP (Ch2) were sequentially acquired on the ArrayScan V[TI] 10x using the XF100 excitation and emission filter set
10	Assay readout	MCRAID-Ch2	Images were analyzed using the MT image analysis algorithm using the mean circ (nucleus)–ring (cytoplasm) average intensity difference to quantify the AR-GFP localization

ASSAY and Drug Development Technologies. **2016**, *14*, 226-239

High-throughput High-content Screening (HCS)

❖ High-throughput screening of an NIH library of 219,055 compounds



A. Scatterplot of the mean circ – ring average intensity difference intensity data from a single representative HCS assay plate

B. Overlay scatterplot of the normalized % inhibition data from 30x384-well assay plates from a representative screening operation run

B. Binned results frequency distribution graph of the normalized % inhibition data from the primary HCS

ASSAY and Drug Development Technologies. **2016**, *14*, 226-239

High-throughput High-content Screening (HCS)

- ❖ GFP-AR Nuclear Localization HCS Campaign Summary
- \- Eliminated cytotoxic and autofluorescent compounds
- \- 182 compounds confirmed to have reproducible inhibition >50%
- \- 163 compounds have IC_{50}: <40 μM
- \- Elimination of compounds: (i) that often interfere with HTC assay, (ii) involved in other signaling pathways, (iii) <95% purity, (iv) IC_{50}: >10 μM
- \- 23 compounds were subjected to further analysis

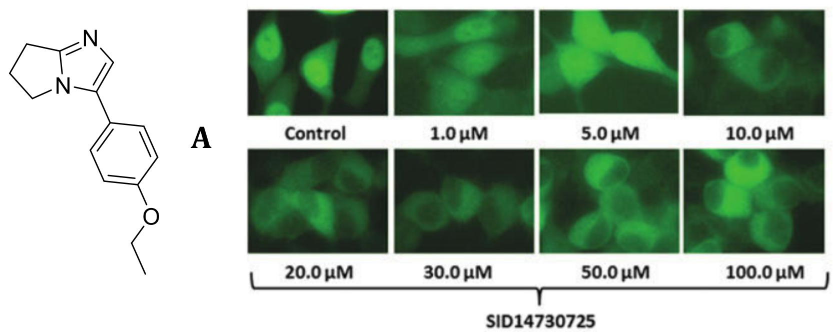
HCS phase and category	Number of compounds	% of total
Primary screen		
NIH MLSCN compound library	219055	100
Cytotoxic outliers, SOCb zsc <−4	828	0.38
Ch1 Fld outliers, MNTIe and MNAIf zs >4	2,591	1.18
Ch2 FI outliers, MCTIg, MRTIh, MRAIi zs >4	150	0.07
Primary HCS actives,% inhibition MCRAIDj >60%	980	0.45
Active confirmation		
Mean% inhibition MCRAID >50% (n=3)	182	18.6 (980)
IC_{50} hit confirmation		
IC50 < 1 μM	2	1.1 (182)
IC50 > 1 but <10 μM	65	35.7 (182)
IC50 > 10 but <40 μM	96	52.7 (182)
IC50 > 40 μM	19	10.4 (182)

NIH molecular library screening center network (MLSCN) compound library. FI, fluorescence intensity; MCRAID, mean circ–ring average intensity difference Ch2; MCTI, mean "circ" total intensity Ch2; MNAI, mean nuclear average intensity Ch1; MNTI, mean nuclear total intensity Ch1; MRAI, mean "ring" average intensity Ch2; MRTI, mean "ring" total intensity Ch2; NIH, National Institutes of Health; SOC, selected object/cell count per image; zs, z-score.

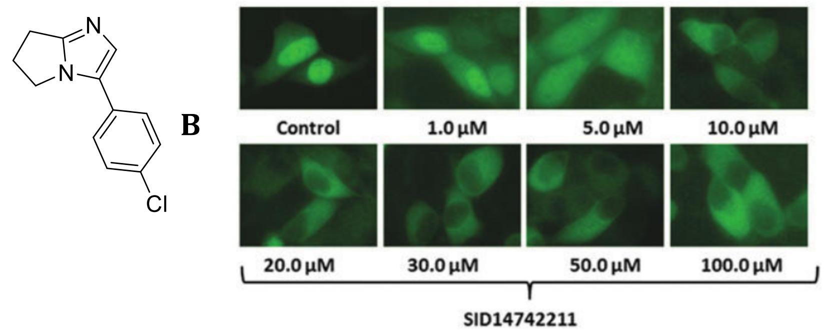
ASSAY and Drug Development Technologies. **2016**, *14*, 226-239

High-throughput Screening (HCS)

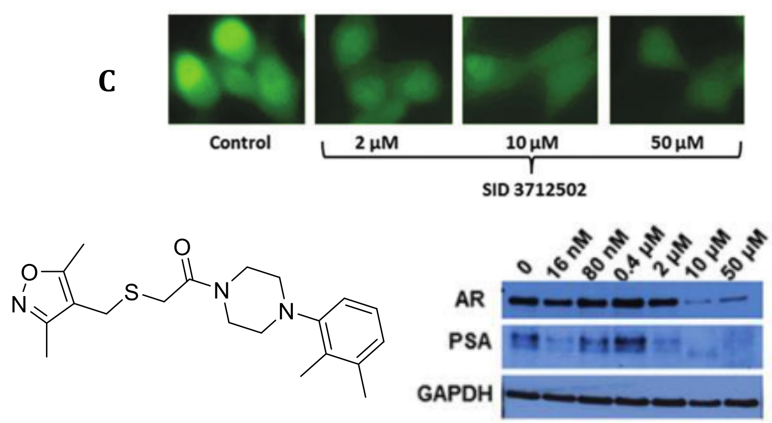
A

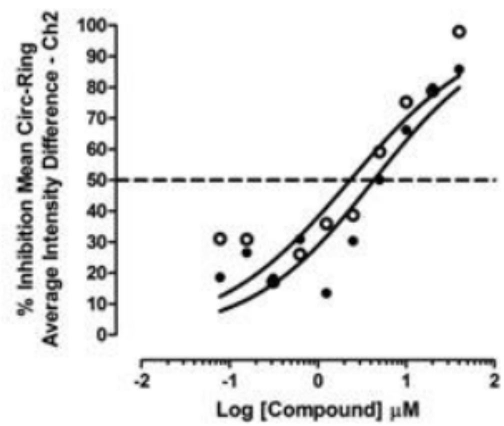


B



C





ASSAY and Drug Development Technologies. **2016**, *14*, 226-239

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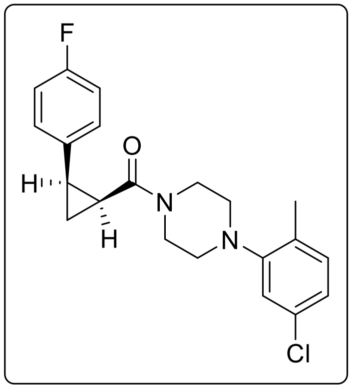
Wipf Group Strategy

Discovery of JJ450



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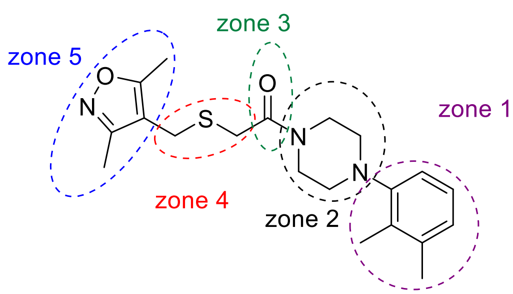
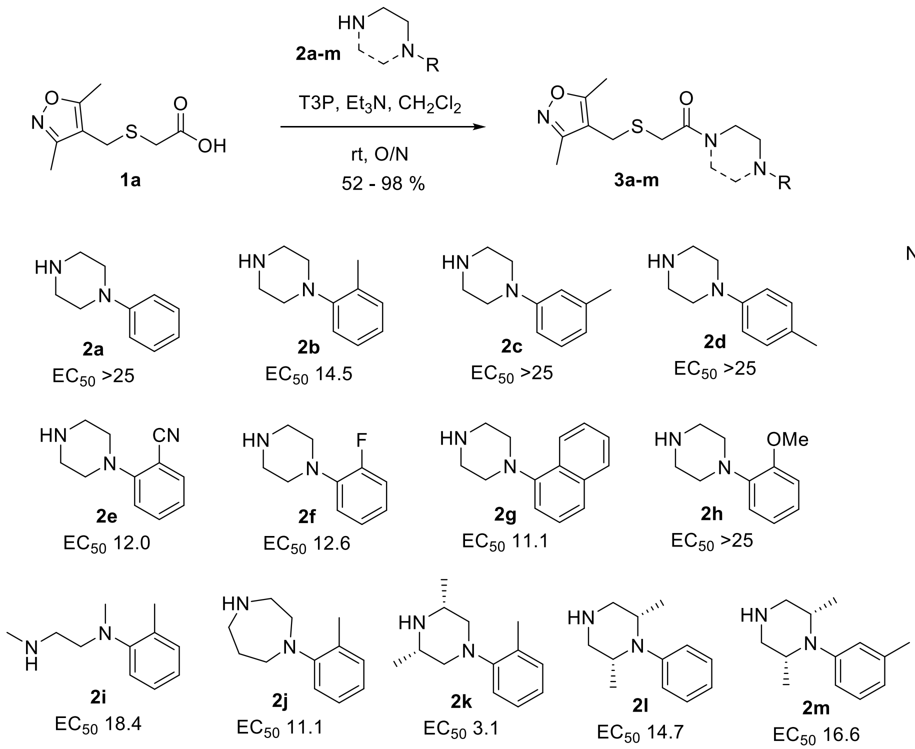
Zones of Structural Modifications

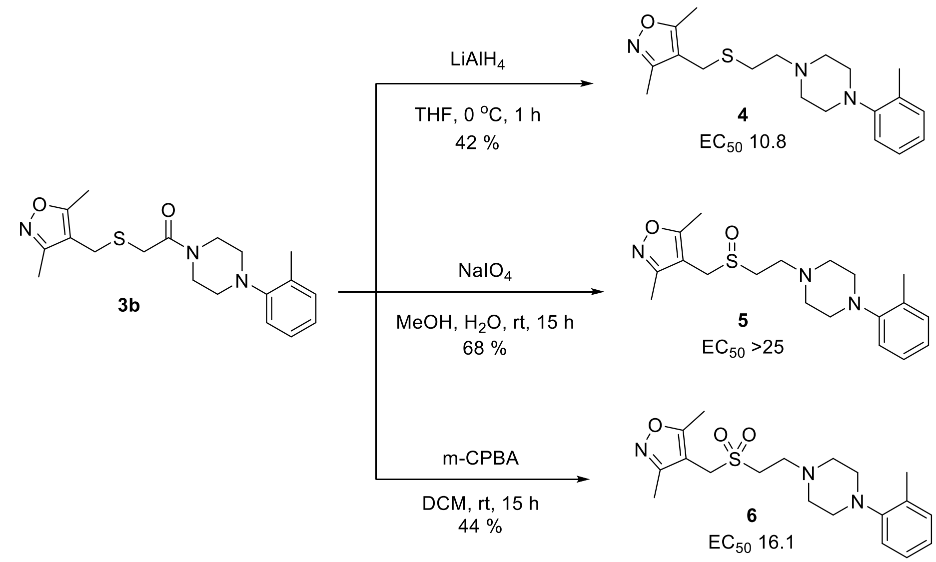


- ❖ Zone 1: ortho-substituent on the phenyl ring is important for activity
- ❖ Zone 2: sterically bulky piperazine is superior to flexible, unsubstitued or bridged analogues
- ❖ Zone 3: carbonyl is not required; sulfonamide or amine are tolerated
- ❖ Zone 4: thioether oxidation reduced activity; cyclopropane significantly improved EC_{50}
- ❖ Substituted phenyl groups are equipotent with 3,5-dimethylisoxazole

ACS Med. Chem. Lett. **2016**, *7*, 785-790

Synthesis of DMI-containing Analogs

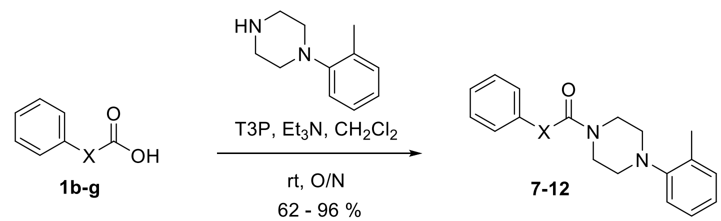


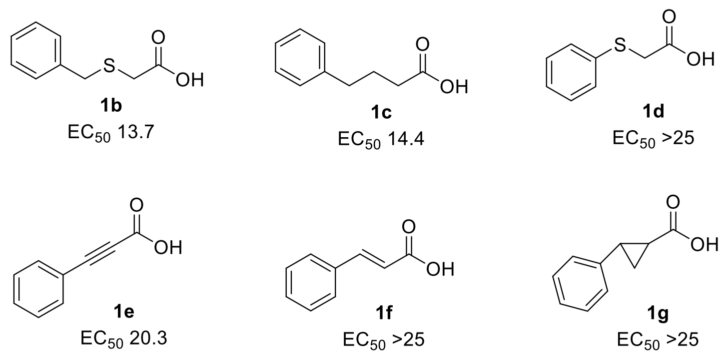


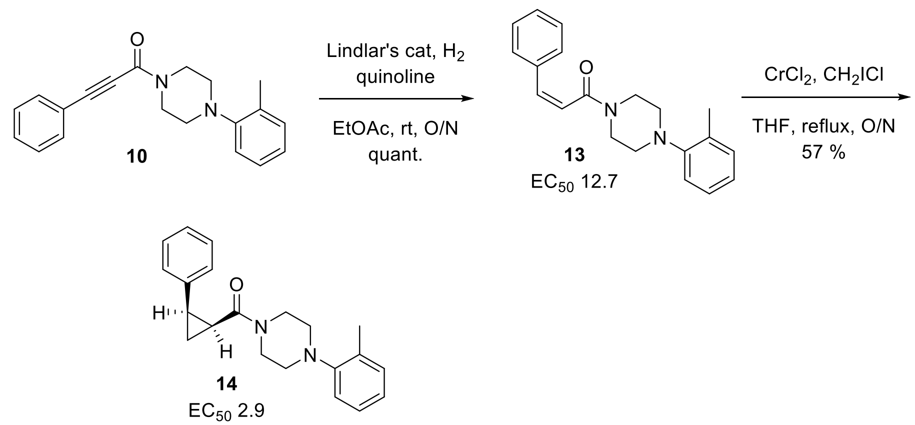
ACS Med. Chem. Lett. **2016**, *7*, 785-790

**EC50 values reported in μM*

Synthesis of Piperazine Analogs







ACS Med. Chem. Lett. **2016**, *7*, 785-790

**EC50 values reported in μM*

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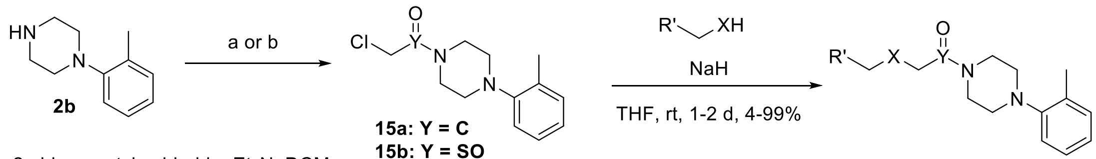
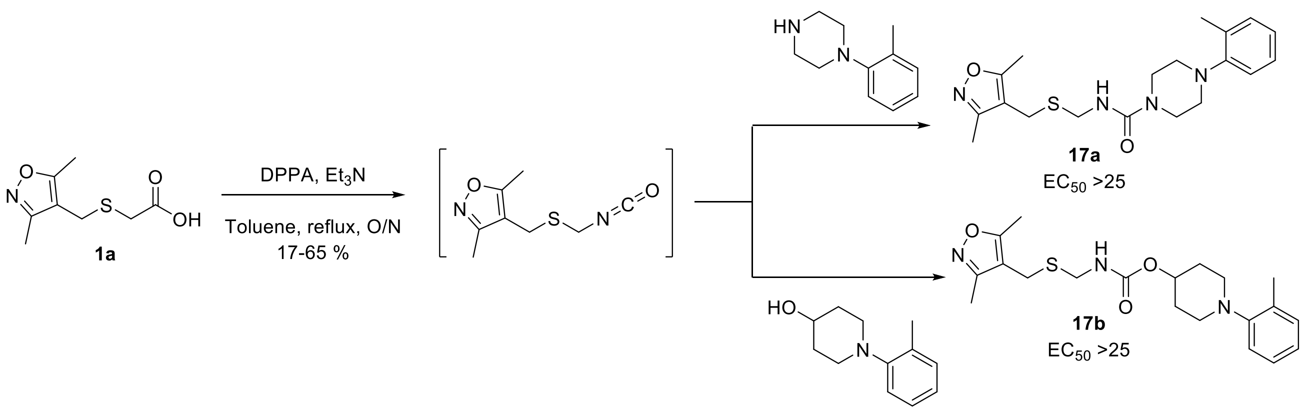
Synthesis of Heteroatom Analogs



a. 2-chloroacetyle chloride, Et_3N, DCM, rt, O/N, 99 %
b. chloromethanesulfonyl chloride, Et_3N, DCM, rt, O/N, 85 %

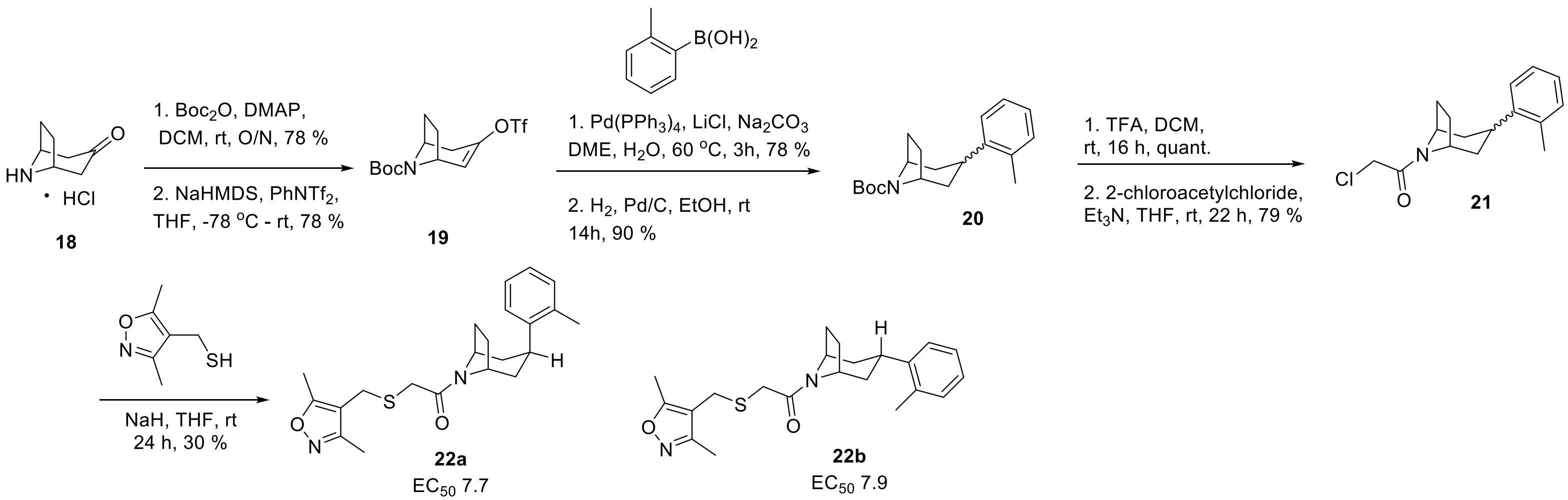
	Y	X	R'	EC50
16a	C	O	3,5-DMI	>25
16b	C	NMe	Ph	>25
16c	SO	S	3,5-DMI	7.2



ACS Med. Chem. Lett. **2016**, *7*, 785-790

**EC50 values reported in μM*

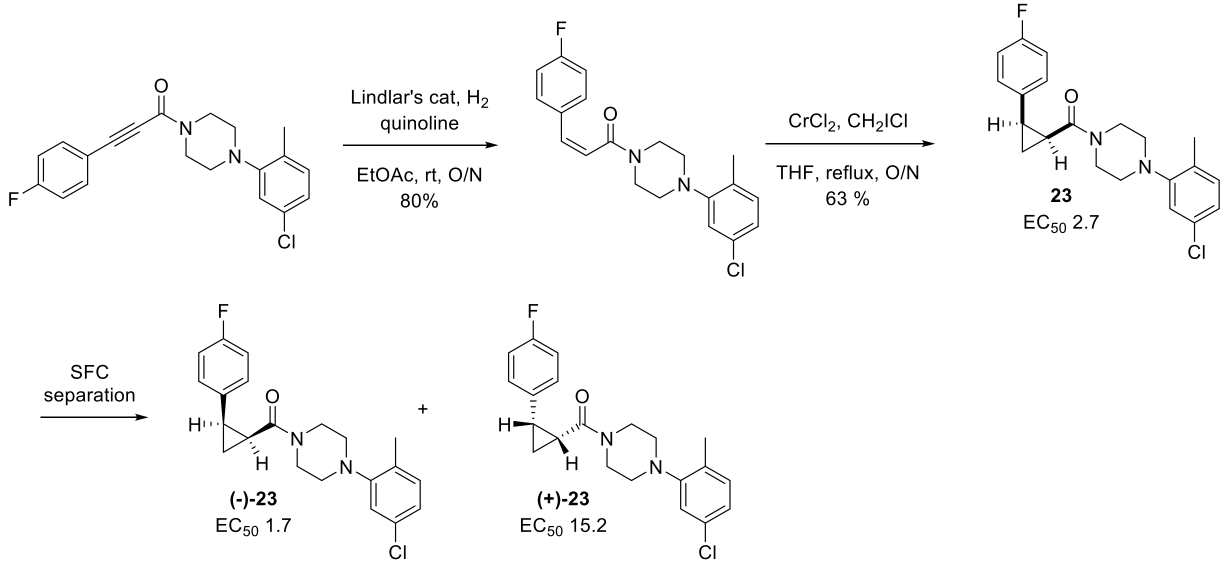
Synthesis of Bridged Analogs



ACS Med. Chem. Lett. **2016**, *7*, 785-790

**EC50 values reported in μM*

Synthesis of JJ450



ACS Med. Chem. Lett. **2016**, *7*, 785-790

**EC50 values reported in µM*

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Wipf Group Strategy

Current Effort

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Conclusions

- A total of 85 new analogs have been synthesized in our lab
- Development of scaffolds to improve potency and metabolic stability
- Development of a more efficient enantioselective synthesis of analogs
- Understanding the mechanism of action would be very helpful in designing more future analogs

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Acknowledgments

- Dr. Peter Wipf
- James Johnson
- Dr. Keita Takubo
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- John Milligan
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- Funding obtained from DOD & UPMC Enterprise

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